

## Induction of Stress Proteins in Tomato Leaves and Structure of Gene Encoding Stress Protein

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### Summary

Incubation of tomato leaves with salicylic acid or infection with tobacco mosaic virus were found to induce three new type of structurally unfamiliar proteins. The molecular weights of these stress proteins were 32k (SP32), 23k (SP23) and 14k daltons (SP14). These proteins were purified and the amino acid sequences of N terminus were determined. The enzyme activity of SP32 was examined and the SP32 possessed both chitinase and  $\beta$ -1, 3-glucanase activities. The mRNAs from salicylic acid treated leaves were isolated and cDNA clones were screened. A gene encoding SP32 were selected from cDNA library and the cloned SP32 gene were sequenced.

Key words: *Lycopersicon esculentum*, gene structure, stress proteins, salicylic acid, pathogenesis-related protein.

### Introduction

Some plants produce a set of new proteins in response to viral infection<sup>17)</sup>, and these proteins, called pathogenesis-related (PR) proteins, consist of 10 different proteins in *Nicotiana tabacum*<sup>16)</sup>. These proteins were also induced by incubation with some chemicals such as salicylic acid<sup>1, 13)</sup>. Thus, the protein should be called as stress protein (SP). In tomato variety Rutgers, Rentita and Hilda 72, the PR proteins were newly synthesized by infection with tobacco mosaic virus (TMV), and the molecular weight (MW) of a major PR protein (p14) was 14k daltons<sup>4, 5)</sup>. The complete sequence of amino acid residues of the p14 was determined<sup>12)</sup>. We also tried to purify these SP or PR proteins, using tomato (*Lycopersicon esculentum* cv. Red-pair) plants.

One of presumed functions of PR proteins was thought to be digestion of viral or fungal cell wall, and some PR proteins have chitinase<sup>10)</sup> or  $\beta$ -1, 3-glucanase activity<sup>8)</sup>. Therefore we examined these activities in the SP.

The nucleotide sequence of genes encoding PR proteins in tobacco were previously reported<sup>6, 14)</sup>. We also determined the sequence of gene encoding the SP and analyzed sequence homology with tobacco PR-genes.

## Materials and Methods

### Induction of stress proteins

Plants of *Lycopersicon esculentum* cv. Red-pair were cultivated in greenhouse for 3 months and the discs (10 mm in diameter) from fully expanded leaves were floated on water with 0.1 % salicylic acid (SA) for 48 hr. In the case of TMV infection, the leaf discs dusted with carborandum (6,000 mesh) were inoculated for 24 hr with a solution of purified TMV (10  $\mu$ g/ml).

### Protein extraction

The SA-treated or TMV-infected leaf discs were homogenized with 4 volume of 84 mM citric acid-32 mM sodium phosphate buffer, pH 2.8, containing 14 mM 2-mercaptoethanol and 6 mM sodium ascorbate. The homogenate was squeezed through two layers of cheesecloth and filtrate was centrifuged for 20 min at 20,000 $\times$ g. The supernatant fluid was used as the crude protein solution. For polyacrylamide gel electrophoresis (PAGE), the solution was dialyzed against 5 mM Tris-HCl buffer, pH 8.3. Protein content was determined by the method of Lowry et al.<sup>11)</sup> with bovine serum albumin as the standard.

### Purification of stress proteins

The crude protein solution was passed through a Sephadex G-25 column (6 $\times$ 40 cm) previously equilibrated with the pH 2.8 buffer to remove polyphenols and other low molecular weight substances. The elute was fractionated with ammonium sulfate, and the precipitate by 35 % and 80 % saturation was dissolved in 10 ml of 50 mM sodium phosphate-1 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.0, containing 6 mM sodium ascorbate. After dialysis against same buffer, the sample was applied to ion exchange chromatography (Pharmacia, Uppsala, Sweden; FPLC MonoQ column) previously equilibrated with pH 8.0 buffer. The elute by pH 8.0 buffer with 150 mM NaCl was examined with PAGE and fractions contained SP were isolated. These fractions were dialysed against 50 mM sodium phosphate-150 mM NaCl buffer, pH 7.0, the samples were applied to gel permeation chromatography (Pharmacia, FPLC Superose column) previously equilibrated with same buffer, and the elute was examined by PAGE for its purity.

### Polyacrylamide gel electrophoresis

Electrophoresis on 10 % polyacrylamide slab gels (13.5 $\times$ 15 mm) in a nondenaturing buffer was performed as described by Davis<sup>7)</sup> (native-PAGE). Electrophoresis on 15 % polyacrylamide slab gels containing 0.1 % sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli<sup>9)</sup> (SDS-PAGE). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 or silver-stain to make the protein bands visible.

### Assay for chitinase activity

The purified SP32 was used as materials for measurement of chitinase activity.

For colorimetric assay of chitinase, the reaction mixture (in 0.5 ml enzyme) contained 1 mg colloidal chitin, 0.3  $\mu$ M sodium azide, and 14  $\mu$ M sodium acetate buffer (pH 4.5). It was incubated at 37 °C for 2 hr and the reaction was stopped by centrifugation (1,000 $\times$ g, for 2 min); 0.3 ml of the supernatant were incubated at 37 °C with 0.02 ml of 3 % helicase and 0.03 ml of 1 M potassium phosphate buffer (pH 7.1) to hydrolyze the chitin oligomers<sup>3)</sup>.

The resulting N-acetylglucosamine (GlcNAc) was determined according to the method of Reissig et al.<sup>15)</sup>, using internal standards of GlcNAc in the assay mixtures for the calculations. Enzyme and substrate blanks were also included. Formation of a reaction product was non-linearly related to the enzyme concentration. Therefore, a dilution series of the enzyme was prepared and the activity was determined for enzyme concentrations approaching zero. The amount of enzyme producing 1 M/sec GlcNAc equivalents at infinite dilution was defined as a Katal (kat).

#### **Assay for $\beta$ -1, 3-glucanase activity**

Activity of  $\beta$ -1, 3-glucanase was measured using the purified SP32, and assayed by measuring the rate of reducing sugar production with laminarin as the substrate. The assay mixture consisted of 0.5 ml of 0.1 M acetate buffer (pH 5.2) containing 1 mg/ml laminarin and of various volumes of enzymatic solution. After a 30 min incubation at 37 °C, 0.5 ml of the alkaline copper reagent was added<sup>2)</sup> and the mixture was heated at 100 °C for 15 min. After cooling, 0.5 ml of Nelson's chromogenic reagent was added and the absorbance measured at 660 nm<sup>2)</sup>. Standards of glucose and enzyme or substrate blanks were included. A Katal (kat) was defined as the enzyme activity catalysing the formation of 1 M glucose equivalents/sec.

#### **Amino acids sequence determination of N terminus of stress proteins**

Purified SP32, SP23 and SP14 were used for determination of amino acids sequences of their N terminus with an automatic sequenator (Applied Biosystems, Foster City, USA; 477A).

#### **cDNA cloning and sequencing of gene encoding SP32**

A cDNA library was prepared using RNA isolated from tomato leaves incubated with 0.1 % SA. Double stranded cDNA produced from size-fractionated poly (A)<sup>+</sup> RNA was inserted into the BamH I site of Charomid 9-36 (Nippon Gene, Tokyo, Japan) by a homopolymeric dC-dG tailing method and transformed into *Escherichia coli* host strain DH1. The cDNA library was first screened by differential colony hybridization. As a source of the DNAs used to produce radioactive probes, nucleotide sequences presumed by amino acids sequence of SP32 were synthesized using DNA synthesizer (Pharmacia, Gene Assembler Plus) and labelled with [<sup>32</sup>P]. The 132 positive clones screened by colony hybridization gave a stronger hybridization signal with radioactively labelled cDNA, the length of inserted cDNA was measured with agarose gel electrophoresis, and 12 clones containing cDNAs more than 900 base pairs were selected.

The pSP32- $\gamma$  (inserted cDNA length was about 1100 base pairs) was selected for nucleotide sequence determination. The sequence was determined using the dideoxynucleotide chain-termination method.

## **Results and Discussion**

#### **Purification of stress proteins**

Soluble proteins were extracted from 0.1 % SA treated tomato leaves and analyzed with native-PAGE and SDS-PAGE. Three new proteins were synthesized and their MWs

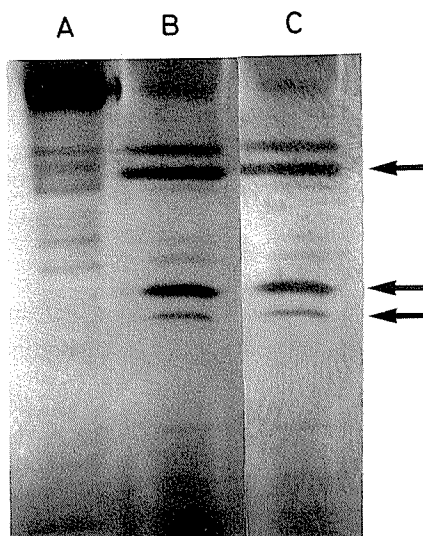


Fig. 1 Electrophoretic analysis of crude extracts from tomato leaves. Tomato leaves were incubated with water for 48 hr (A) or 0.1 % salicylic acid for 48 hr (B) or inoculated with 10  $\mu$ g/ml of purified TMV for 24 hr (C), and homogenized with 84 mM citric acid-32 mM sodium phosphate buffer, pH 2.8, containing 14 mM 2-mercaptoethanol and 6 mM sodium ascorbate. The homogenate was centrifuged for 20 min at 20,000 $\times$ g, the supernatant fluid was dialysed against 5 mM Tris-HCl (pH 8.3) and the solution was analysed native-PAGE. The gels were stained with silverstaining. The newly synthesized proteins are indicated by arrows.

Table 1 Fractionation of crude tomato proteins by ammonium sulfate (AS) precipitation.

Fraction	Protein amount (g)	SP14	SP23	SP32
Crude extract	8.1	+	+	+
35 % AS sup	5.7	+	+	+
35 % AS ppt	1.9	—	—	—
80 % AS sup	1.7	—	—	—
80 % AS ppt	3.4	+	+	+

Tomato leaves (1 kg fresh weight) incubated in 0.1 % SA solution were homogenized with 84 mM citric acid-32 mM sodium phosphate buffer, pH 2.8, containing 14 mM 2-mercaptoethanol and 6 mM sodium ascorbate. The homogenate was centrifuged for 20 min at 20,000 $\times$ g, and the supernatant fluid was as crude extract. The crude extract was passed through a Sephadex G-25 column and the elute was fractionated with ammonium sulfate. Ammonium sulfate at 35 % saturation was added to the elute, incubated for 1 hr, and then centrifuged for 20 min at 20,000 $\times$ g. Ammonium sulfate at 80 % saturation was added to the resulted supernatant fluid, incubated for 1 hr, and then centrifuged for 20 min at 20,000 $\times$ g. The protein amount of each fraction was measured, and the presence of SPs was detected by native-PAGE.

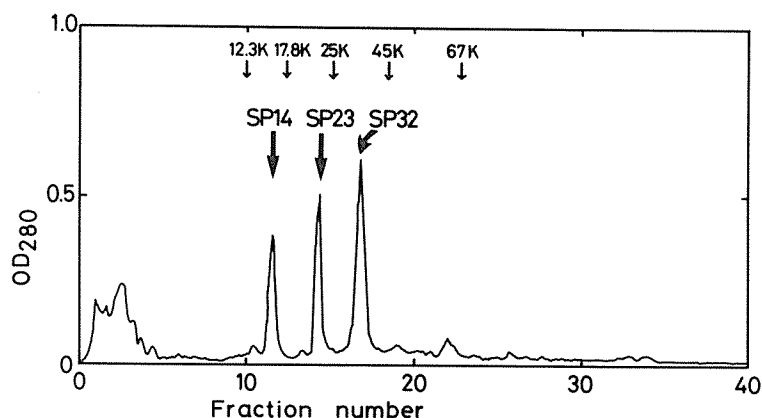


Fig. 2 Gel permeation chromatography profile of tomato stress proteins. Positions of SP14, SP23 and SP32 were indicated. Marker proteins used for detection of MWs were cytochrome C (12.3k), myoglobin (17.8k), chymotrypsinogen A (25k), egg albumin (45k) and bovine serum albumin (67k).

were 32k, 23k and 14k daltons (Fig. 1). Each proteins named SP32, SP23 and SP14, respectively.

In the case of infection with TMV, similar SPs were induced (Fig. 1). The crude protein solution was fractionated with ammonium sulfate, and the supernatants or precipitates with 35 % and 80 % saturation were examined by native-PAGE. As shown in Table 1, all of SP were fractionated in the supernatants of 35 % ammonium sulfate and the precipitates of 80 % ammonium sulfate. After application with ion exchange chromatography, SP fractions were applied to gel permeation chromatography and the sharp 3 peaks were appeared in the elution profile (Fig. 2). The total purification step of SP was summarized in Table 2.

Incubation with SA induced 3 SPs and these SPs were also synthesized by infection with TMV (Fig. 1). This result seems to show that SA and TMV had similar function for SP (or PR protein) synthesis. In fact, some PR proteins were induced by SA treatment

Table 2 Purification of SPs from SA-treated tomato leaves.

Fraction	Total Protein (mg)	SP14 (mg)	SP23 (mg)	SP32 (mg)	Recovery of SPs <sup>a</sup> (%)
Crude extract <sup>b</sup>	8,100	300	700	950	100
35 % AS <sup>c</sup> sup	5,700	250	500	650	71.8
80 % AS ppt	3,400	200	400	500	56.4
Ion exchange chromatography	1,800	150	350	400	46.2
Gel permeation chromatography	800	100	250	350	35.9

<sup>a</sup>Recovery of SPs were calculated with the amounts of SP14+SP23+SP32.

<sup>b</sup>Crude extract was prepared from 1 kg of SA-treated tomato leaves.

<sup>c</sup>AS means ammonium sulfate.

in tobacco leaves<sup>1, 13)</sup>.

In tomato cv. Rutgers, Rentia and Hilda 72, infection with viroid, virus and fungus induced nine PR proteins with the MWs of 10k, 11k, 12k, 13k, 14k, 25k, 31k, 33k and 38k daltons, and most dramatic change was observed in the case of the major PR protein with the MW of 14k dalton called p14<sup>4, 5)</sup>. In our experiments (tomato cv. Red-pair), although SP with the MW of 14K dalton (SP14) was induced by SA treatment, its amount was relatively low and our major SP was SP32 (Table 2).

#### Amino acids sequences of N terminus of three SPs

Amino acids sequences of SP14, SP23 and SP32 were examined. The 1st to 16th amino acids in SP14 (Fig. 3), the 1st to 20th amino acids in SP23 (Fig. 4) and the 1st to 22nd amino acids in SP32 (Fig. 5) were determined. These sequences of 3 SPs were not homologous.

In previously reported tomato PR protein (p14), total amino acids sequence was determined<sup>12)</sup>. In comparison of SP14 with p14, although N terminus and the 3rd amino acid were glutamine in SP14, amino acids in p14 were pyrrolidone carboxylic acid (PCA) and serine, respectively, and other 14 amino acids were same as both SP14 and p14 (Fig. 3). As the PCA was a cyclisation product of glutamine, major amino acids

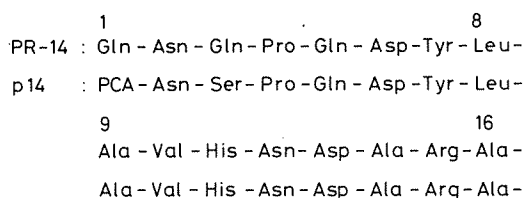


Fig. 3 Amino acid sequence of N terminus in SP14. Amino acid sequence of tomato PR protein (p14)<sup>12)</sup> was also shown.

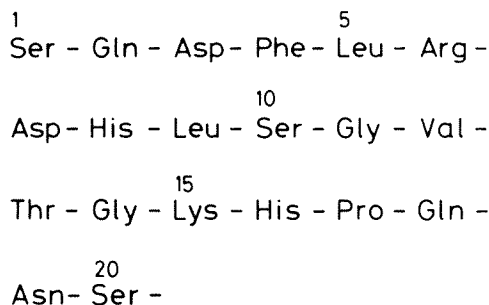


Fig. 4 Amino acid sequence of N terminus in SP23.

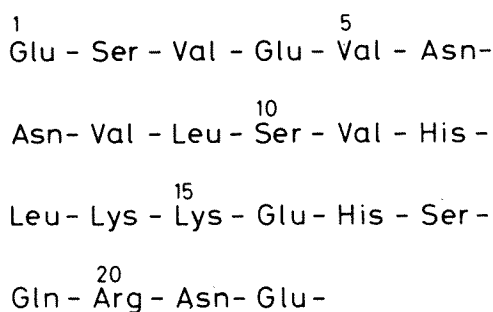


Fig. 5 Amino acid sequence of N terminus in SP32.

Table 3 Chitinase activity of SP32.

Amount of SP32 ( $\mu$ g)	Chitinase activity (nkat/mg protein)
0	0.2
0.5	8.4
1	36.4
2	24.8

Chitinase activities of various amount of the purified SP32 were measured as described in Materials and Methods.

Table 4  $\beta$ -1, 3-Glucanase activity of SP32.

Amount of SP32 ( $\mu$ g)	$\beta$ -1, 3-glucanase activity (nkat/mg protein)
0	110
0.5	660
1	1,450
2	1,300

$\beta$ -1, 3-Glucanase activities of various amount of the purified SP32 were measured as described in Materials and Methods.

sequences of SP14 and p14 seemed to be almost same.

### Enzyme activity of SP32

The activities of chitinase and  $\beta$ -1, 3-glucanase were assayed using purified SP32. High activity of chitinase was detected, and the activity of 1  $\mu$ g SP32 was 180 times higher than basic activity (Table 3). In the case of  $\beta$ -1, 3-glucanase, relatively high activity was detected, and the activity of 1  $\mu$ g SP32 was 13 times higher than basic activity (Table 4).

When TMV infected the tobacco leaves, four proteins with chitinase activities were synthesized and their MWs were 27.5k, 28.5k, 32k and 34k daltons<sup>10</sup>. Two chitinases with MWs of 27.5k and 28.5k daltons were thought to be PR proteins, and a chitinase with MW of 32k dalton exhibited higher specific enzyme activity than two PR proteins<sup>10</sup>. The chitinase and our SP32 in tomato showed same MW.

In tobacco leaves, four PR proteins have  $\beta$ -1, 3-glucanase activity, these MWs were 26k, 29k, 32k and 34k daltons and two proteins with MWs of 32k and 34k daltons exhibited high specific activities<sup>9</sup>. In this case, the  $\beta$ -1, 3-glucanase with MW of 32k dalton was also exhibited same MW with our SP32.

According to the above results, it is possible that chitinase and  $\beta$ -1, 3-glucanase with MW of 32k dalton and SP32 were same proteins. In fact, chitinase and  $\beta$ -1, 3-glucanase with same MW were regulated co-ordinately by ethylene in bean leaves<sup>18</sup>.

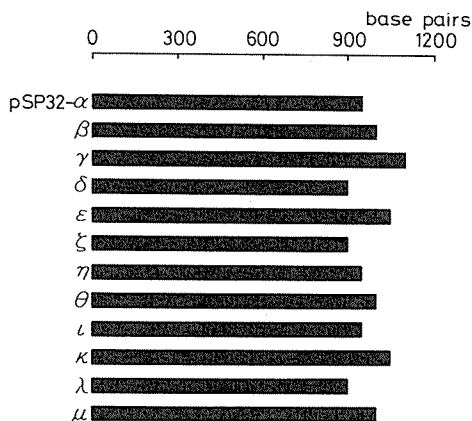


Fig. 6 Length of inserted cDNA in cDNA library. Clones inserted more than 900 base pairs were shown.

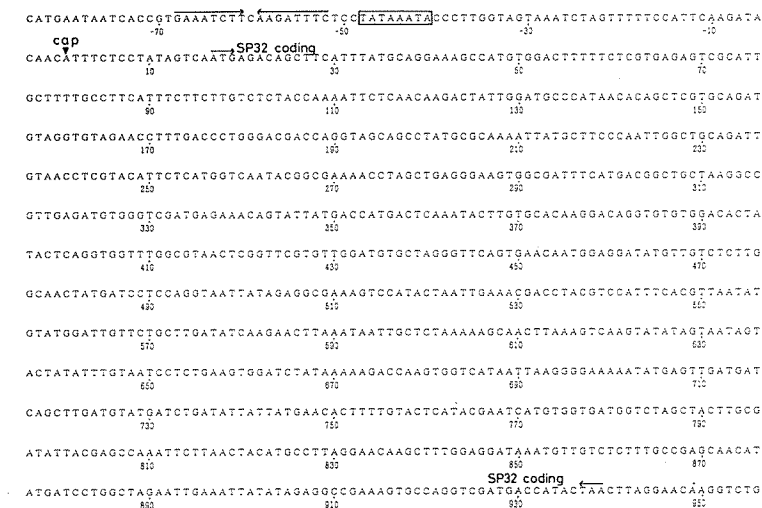


Fig. 7 Nucleotide sequence of pSP32- $\gamma$ .

### Nucleotide sequence of gene encoding SP32

According to the amino acids sequence of N terminus of SP32, probe nucleotides (TTTTTTCTTGTAAG, TTCTTTCTTGTAAG, TTTTTCCTTGTAAG or TTTTTTCTTGAG) were synthesized. From the results obtained by colony hybridization, the positive 132 clones were screened. Using these clones, the length of inserted cDNA was measured and the results showed that the 12 clones contained cDNA more than 900 base pairs (Fig. 6). The clone (pSP32- $\gamma$ ) inserted cDNA about 1,100 base pairs was used as material for nucleotide sequence determination and the result was shown in Fig. 7. The SP32 coding region was 923 base pairs and its GC-content was 41 %. The sequence TATAAATA was found at position -48 corresponding to the TATA box. A perfect dyad symmetrical structure was detected just upstream of this sequence.

The nucleotide sequence of SP32 were compared with previously reported sequences of tobacco PR protein (PR1a)<sup>6, 14</sup>, and SP32 coding region showed only 25.6 % homology with PR1a sequence.

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## トマト葉におけるストレス蛋白質の誘導と その遺伝子の構造

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### 摘 要

トマトの葉をサリチル酸で処理するかあるいはタバコモザイクウイルスに感染させると3種の新しい蛋白質が誘導されることを見いだした。それらのストレス蛋白質の分子量はそれぞれ32 k (SP32), 23 k (SP23), 14 k (SP14) ダルトンであった。この3種のストレス蛋白質のN末端のアミノ酸配列を決定した。さらにSP32はキチネースと $\beta$ -1, 3-グルカネースの両者の酵素活性を有していた。サリチル酸処理葉からmRNAを単離し、cDNAを得た。cDNAライブラリーよりSP32の遺伝子を選抜しその塩基配列を決定した。